

Alkyl lysophospholipids inhibit phorbol ester-stimulated phospholipase D activity and DNA synthesis in fibroblasts

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Abstract The antineoplastic alkyl lysophospholipids (ALP) 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH₃) and 1-*S*-hexadecylthio-2-methoxymethyl-2-deoxy-*rac*-glycero-3-phosphocholine (BM41.440) were found to alter phospholipase D (PLD)-mediated phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) hydrolysis in NIH 3T3 fibroblasts. After a shorter (50 min) treatment, 2.5–7.5 µg/ml concentrations of ALP stimulated PtdCho, but not PtdEtn, hydrolysis 2–4-fold. At the same time, 7.5–25 µg/ml concentrations of ALP significantly inhibited the larger (5.8–6.5-fold) stimulatory effects of phorbol 12-myristate 13-acetate (PMA) on both PtdCho and PtdEtn hydrolysis. When a brief (30 min) exposure of cells to 1–2.5 µg/ml concentrations of BM41.440 was followed by incubation of washed cells for 3–16 h prior to the assay of PLD activity or DNA synthesis, the treated cells exhibited no increased PtdCho hydrolysis, while their responses to the stimulatory PMA effects on both PLD activity and DNA synthesis were strongly reduced. The results suggest that the PLD and protein kinase C systems may be important cellular targets of ALP actions.

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1. Introduction

The alkyl lysophospholipids (ALP) 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH₃) and 1-*S*-hexadecylthio-2-methoxymethyl-2-deoxy-*rac*-glycero-3-phosphocholine (BM41.440) represent prototypes of anti-tumor ether lysophospholipids [1–4]. ALP do not directly affect DNA synthesis and their most likely target is the plasma membrane. Accordingly, ALP have been shown to inhibit several important membrane-associated activities, including de novo phosphatidylcholine (PtdCho) synthesis [5], Na⁺, K⁺-ATP-ase and sodium pump activity [6,7], epidermal growth factor binding [8], arachidonate release by phospholipase A₂ [9], phosphatidylinositol-specific phospholipase C activity [10], as well as calcium signaling [11]. ALP also inhibit the activities of phosphatidylinositol-3'-kinase [12] and p42/p44 mitogen activated kinases [13], but they stimulate expression of *fos* and *jun* proto-oncogenes [14].

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Abbreviations: ET-18-OCH₃, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; BM41.440, 1-*S*-hexadecylthio-2-methoxymethyl-2-deoxy-*rac*-glycero-3-phosphocholine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdEtOH, phosphatidylethanol

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ALP have also been found to inhibit protein kinase C (PKC) activity in vitro through competitive inhibition of phosphatidylserine binding to the regulatory domain of calcium-dependent PKC isozymes [15,16], and to inhibit phorbol 12-myristate 13-acetate (PMA)-induced protein phosphorylation in intact HL60 cells [17]. However, the biological significance of PKC inhibition by ALP is presently unclear.

Major targets of the PMA activated PKC system includes phospholipase D (PLD) acting on PtdCho [18–22] and/or phosphatidylethanolamine (PtdEtn) [23]. Since some of the primary and secondary products of PtdCho and PtdEtn hydrolysis, including phosphatidic acid, 1,2-diacylglycerol, ethanolamine and phosphocholine, can regulate cell growth [reviewed in ref. [24]], it was of interest to determine the effects of ALP on the hydrolysis of these phospholipids.

In the present study, we have therefore examined the effects of ET-18-OCH₃ and BM41.440 on PLD-mediated phospholipid hydrolysis in NIH 3T3 fibroblasts which express both the PtdCho- and PtdEtn- hydrolyzing PLD activities [25]. Our data show that ALP exert multiple, previously unrecognized, effects on PLD-mediated phospholipid hydrolysis, including transient stimulation of a PtdCho-specific PLD activity and lasting inhibition of PMA-stimulated hydrolysis of both PtdCho and PtdEtn.

2. Materials and methods

2.1. Materials

BM41.440 (Ilmofosine) was a generous gift from Dr. Dieter B.J. Herrmann, Boehringer Mannheim GmbH (Mannheim, Germany). ET-18-OCH₃ was either purchased from Bachem Feinchemikalien (Bubendorf, Switzerland) or was provided by Dr. Wolfgang J. Baumann (The Hormel Institute, Austin, MN). Both compounds were more than 99% pure as determined by thin layer chromatography and carbon-13 NMR. PMA, phosphocholine, sphingosine-1-phosphate and Dowex-50W[H⁺] were purchased from Sigma; insulin was from Boehringer Mannheim. [1-¹⁴C]Palmitic acid (60 mCi/mmol), [methyl-¹⁴C]choline chloride (50 mCi/mmol) and [2-¹⁴C]-ethanolamine (50 mCi/mmol) were bought from Amersham. Phosphatidylethanol (PtdEtOH) was from Avanti Polar Lipids. Tissue culture reagents were purchased from Gibco BRL.

2.2. Cell culture

NIH 3T3 clone-7 fibroblasts were obtained from Dr. Douglas R. Lowy (National Cancer Institute, NIH, Bethesda, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) as described previously [26].

2.3. Measurement of PtdEtOH formation

NIH 3T3 cells were grown in 12-well plates in the presence of [1-¹⁴C]palmitic acid (1 µCi/ml) and 10% fetal calf serum for 24 h. The cultures were washed and incubated in fresh serum-containing medium for 2 h to decrease the amount of unesterified radiolabeled palmitic acid. Finally, the medium was replaced by serum-free medium, and incubations were continued in the presence of 150 mM ethanol for 50 min. ALP were present throughout the incubation,

while PMA was added 20 min after the start of incubation. Incubations were terminated by scraping the cells into 2 ml of ice-cold methanol, followed by rapid transfer of the methanol extract into 2 ml of chloroform. After phase separation, PtdEtOH present in the chloroform layer was separated from other phospholipids on potassium oxalate (1%)-impregnated Silica Gel H plates (Analtech) with chloroform/methanol/acetone/acetic acid/water (50:10:15:10:2, by vol) as developing solvent.

2.4. Determination of PLD-mediated formation of [^{14}C]choline and [^{14}C]ethanolamine from prelabeled phospholipids

For these experiments, attached NIH3T3 cells (grown in 12-well plates) were labeled for 48 h with [^{14}C]choline (1 $\mu\text{Ci}/\text{ml}$) or [^{14}C]ethanolamine (0.75 $\mu\text{Ci}/\text{ml}$). The cells were washed, incubated in serum-free medium for 2 h, washed again, and finally treated with ALP and/or PMA in serum free-medium supplemented with 20 mM choline or 2 mM ethanolamine, as applicable. Supplementation with unlabeled choline and ethanolamine suppressed further metabolism of newly formed radiolabeled choline and ethanolamine [25,27]. Incubations with ALP were carried out for 50 min; when applicable, PMA was added to the cells 20 min after the start of incubation. In other experiments, prelabeled cells were treated first with 1–2.5 μg concentrations of BM 41.440 for 30 min, washed, and then incubated for 3 h in the absence of BM 41.440. Cells were washed again, and then incubated in fresh serum-free medium (containing 20 mM choline or 2 mM ethanolamine, as appropriate) for 50 min in the absence or presence of PMA. In each case, incubations were terminated by adding to the cell cultures 2 ml of ice-cold methanol. Then, the cells were scraped into methanol, and the methanol extract was rapidly transferred into 2 ml of chloroform. After phase separation, radiolabeled choline and ethanolamine were separated on Dowex-50W[H $^{+}$]-packed columns (Bio-Rad Econo columns; 0.75 ml bed volume) using the procedure described by Cook and Wakelam [28] with minor modifications. The initial flow-through (4.5 ml) along with a following 5 or 3.5 ml wash contained glycerophosphocholine and glycerophosphoethanolamine, respectively. Phosphocholine and phosphoethanolamine were eluted by 18 and 15 ml of water, respectively.

Finally, choline and ethanolamine were eluted by 12.5 and 10 ml of 1 M HCl, respectively. The metabolites of radiolabeled choline and ethanolamine were further identified by TLC, and phospholipids were separated as previously described [25].

2.5. Labeling of cellular DNA with [^3H]thymidine

NIH3T3 cells were grown in 12-well tissue culture plates in the presence of 10% serum to about 40% confluency, washed, and then incubated in serum-free medium for 24 h. Cell cultures (≈ 70 –80% confluent) were treated with 1–2.5 $\mu\text{g}/\text{ml}$ concentrations of BM 41.440 for 30 min, washed, and then treated (in serum-free medium) for 16 h with PMA, insulin, phosphocholine, or sphingosine-1-phosphate as indicated in Fig. 4. After treatments, incubations were continued in the presence of [methyl- ^3H]thymidine (1 $\mu\text{Ci}/\text{well}$) for 60 min. The cells were first washed twice with phosphate-buffered saline, then 4 times with 5% trichloroacetic acid, and finally twice with absolute ethanol. The acid-insoluble material was dissolved in 0.3 M sodium hydroxide, and an aliquot was taken to measure DNA-associated ^3H activity in a liquid scintillation counter.

3. Results and discussion

Treatment of NIH 3T3 cells with 2.5–25 $\mu\text{g}/\text{ml}$ concentrations of ET-18-OCH $_3$ or BM 41.440 for 50 min in serum-free medium did not result in detectable changes in the number of trypan blue excluding cells, as determined immediately after the treatments. However, when cells were treated with 2.5, 10 and 25 $\mu\text{g}/\text{ml}$ concentrations of ALP for 30 min and then the ratio of viable and healthy cells was determined 18 h later, 0, 30–40 and 100% of cells were dead, respectively. In agreement with an earlier report [10], these data indicate that NIH 3T3 cells are moderately sensitive to the cytotoxic actions of ALP. Based on these data, we limited the highest concentration of

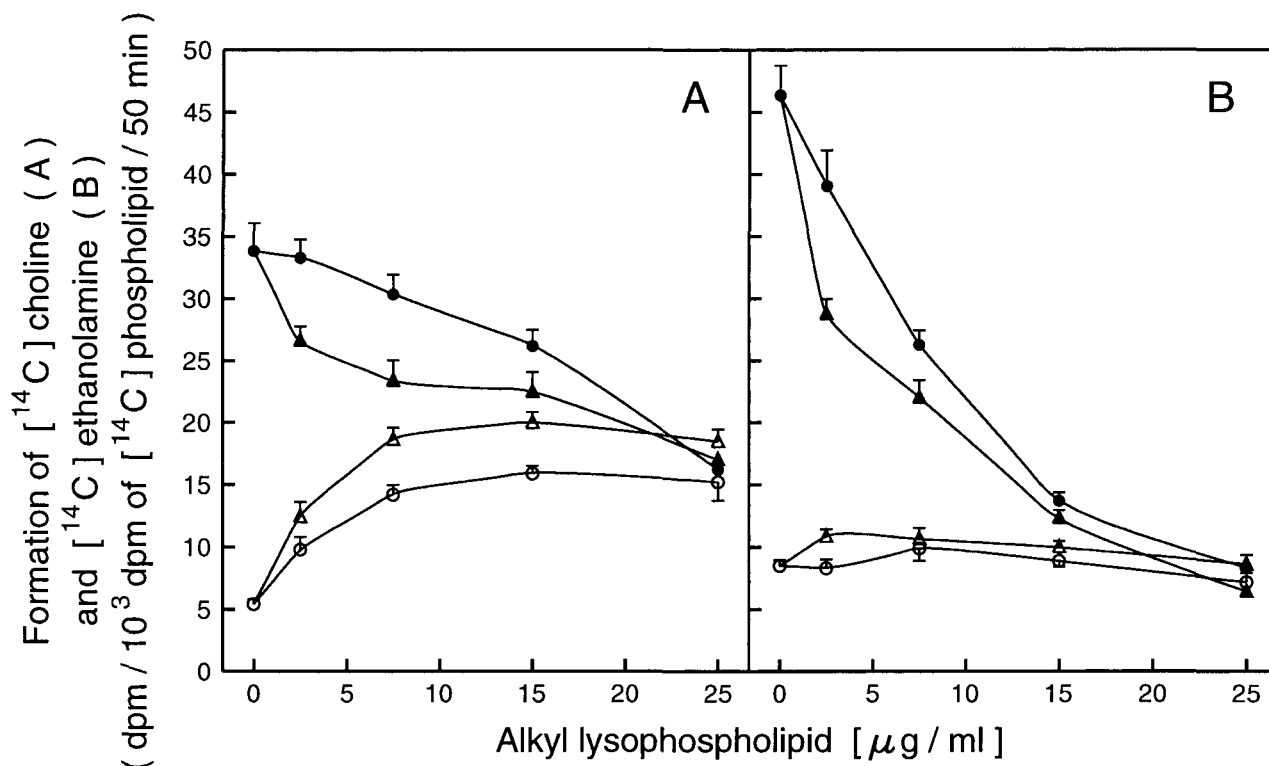


Fig. 1. Effects of ALP on PtdCho and PtdEtn hydrolysis in NIH fibroblasts. NIH 3T3 fibroblasts were labeled with [^{14}C]choline (A) or [^{14}C]ethanolamine (B) for 48 h, followed by treatments with ET-18-OCH $_3$ (○●) or BM 41.440 (△▲) at the indicated concentrations for 50 min; 100 nM PMA was absent (open symbols) or present (closed symbols) during the last 30 min of incubation. Each data point represents the mean \pm S.E.M. of three individual experiments in triplicate.

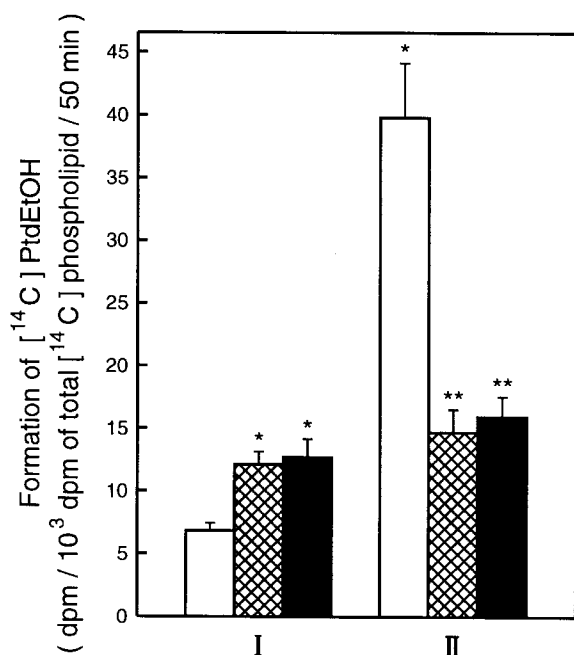


Fig. 2. Effects of ALP on $[^{14}\text{C}]\text{PtdEtOH}$ formation in NIH 3T3 fibroblasts. $[^{14}\text{C}]$ Palmitic acid-labeled NIH 3T3 fibroblasts incubated in the presence of 150 mM ethanol were left untreated (open bars) or treated with 25 $\mu\text{g}/\text{ml}$ of ET-18-OCH₃ (cross-hatched bars) or 25 $\mu\text{g}/\text{ml}$ of BM41.440 (filled bars) for 50 min; 100 nM PMA was absent (I) or present (II) during the last 30 min of incubation. Data represent the mean \pm S.E.M. of three independent experiments in triplicate. Significance of differences from the untreated control (*) or from the effect of PMA alone (**) is indicated by asterisks ($P < 0.05$ – 0.01 ; Student's *t* test).

ALP to 25 and 2.5 $\mu\text{g}/\text{ml}$ in the shorter-term (50 min) and longer-term (3–16 h) experiments, respectively.

Within the 2.5–25 $\mu\text{g}/\text{ml}$ concentration range, both ET-18-OCH₃ and BM41.440 stimulated the formation of $[^{14}\text{C}]\text{choline}$ from $[^{14}\text{C}]\text{PtdCho}$ when incubations were carried out in serum-free medium for 50 min. Nearly maximal (2.6–3.4-fold) and maximal (2.9–3.7-fold) stimulatory effects were observed with 7.5 and 15 $\mu\text{g}/\text{ml}$ concentrations of ALP, respectively (Fig. 1A). In contrast, neither of the ALP enhanced the formation of $[^{14}\text{C}]\text{ethanolamine}$ from $[^{14}\text{C}]\text{PtdEtn}$ (Fig. 1B).

In agreement with our previous finding [25], in NIH 3T3 cells the PMA-activated PLD system hydrolyzed significant amounts of both PtdCho (Fig. 1A) and PtdEtn (Fig. 1B). Both alkyl lysophospholipid analogues inhibited PMA-induced hydrolysis of $[^{14}\text{C}]\text{PtdCho}$ (Fig. 1A) and $[^{14}\text{C}]\text{PtdEtn}$ (Fig. 1B). However, in the case of PtdCho even a high concentration (25 $\mu\text{g}/\text{ml}$) of ALP incompletely inhibited the large stimulatory effect of PMA, reflecting the stimulatory effects of ALP in the absence of PMA (Fig. 1A). By contrast, ALP potently inhibited the stimulatory effects of PMA on PtdEtn hydrolysis (Fig. 1B).

It has been shown that ALP are biologically more active when added to cells in serum-free medium [29]. We also found that in the presence of 10% serum both the stimulatory and inhibitory effects required about 3-times higher concentrations of ALP (data not shown). To facilitate data comparisons with literature data, we continued to use serum-free medium for the following studies.

$[^{14}\text{C}]$ Palmitic acid-labeled cells are suitable to demonstrate

activation of PLD activity, although this method does not allow separate analysis of PtdCho and PtdEtn hydrolysis [23]. After equilibrium labeling of cells with radiolabeled palmitic acid, the ratio of ^{14}C -activity in PtdCho and PtdEtn, on a molar basis, is roughly 2:1. Considering that PtdEtn is significantly labeled with this fatty acid, that its hydrolysis is not stimulated by ALP, and that in unstimulated cells more PtdEtn than PtdCho is degraded, it was expected that ALP would have smaller stimulatory effects on $[^{14}\text{C}]\text{PtdEtOH}$ formation (through the transphosphatidyl reaction) than on $[^{14}\text{C}]\text{choline}$ formation. Indeed, as shown in Fig. 2, ET-18-OCH₃ and BM41.440 (25 $\mu\text{g}/\text{ml}$, each) enhanced $[^{14}\text{C}]\text{PtdEtOH}$ formation 1.8–1.9-fold, which is about half the rate of stimulation observed in case of PtdCho hydrolysis. Nevertheless, these experiments indicated that increased formation of $[^{14}\text{C}]\text{choline}$ from $[^{14}\text{C}]\text{PtdCho}$ in ALP-treated fibroblasts was catalyzed by a PLD activity.

PMA-induced PtdEtOH formation was more effectively inhibited by ALP (Fig. 2) than PMA-induced PtdCho hydrolysis (Fig. 1A). The reason for this discrepancy is that PMA-induced PtdEtn hydrolysis is more strongly inhibited by ALP than PtdCho hydrolysis, and that PtdEtn also is a substrate for PtdEtOH formation.

Subsequent experiments revealed that the cells can be sensitized to the inhibitory effects of ALP by including a longer incubation time between initial ALP treatment and the following PMA treatment. Thus, when $[^{14}\text{C}]\text{choline}$ - or $[^{14}\text{C}]\text{ethanolamine}$ -prelabeled cells were treated with 1–2.5 $\mu\text{g}/\text{ml}$ concentrations of BM41.440 for only 30 min and then incubated for a 3 h period prior to treatments with PMA, BM41.440 considerably inhibited, particularly at the higher (2.5 $\mu\text{g}/\text{ml}$) concentration, PMA-induced hydrolysis of both PtdCho (Fig. 3A) and PtdEtn (Fig. 3B). In the same experiment BM41.440 did not stimulate PtdCho hydrolysis in the absence of PMA (Fig. 1A).

The role of PLD in the mediation of mitogenic effects of PMA and growth factors has not been clarified yet. The emerging view is that PLD activation is one of the important early cellular responses induced by mitogens, which must be followed by other signals for the mitogenesis to proceed [30–32]. In this context, it was of interest to determine how ALP affect mitogenesis induced by PMA and other regulatory agents such as insulin alone or phosphocholine plus sphingosine-1-phosphate in combination. Previously, all these agents were shown to stimulate DNA synthesis in NIH 3T3 cells [33]. As shown in Fig. 4, 1 $\mu\text{g}/\text{ml}$ of BM41.440 had no significant effect on insulin- or phosphocholine plus sphingosine-1-phosphate-induced DNA synthesis, while it inhibited the mitogenic effect of PMA by 70%. At a 2.5 $\mu\text{g}/\text{ml}$ concentration BM41.440 still had no significant effect on insulin-induced DNA synthesis and only modestly (~40%) inhibited the combined effects of phosphocholine and sphingosine-1-phosphate, while it practically abolished the stimulatory effect of PMA (Fig. 4). In another experiment, 2.5 $\mu\text{g}/\text{ml}$ of BM41.440 inhibited the mitogenic effect of 10% serum only by about 20%. These experiments suggest that ALP primarily affects mitogenesis regulated by the PKC system, while the effects of other mitogens are less sensitive to the inhibitory action of ALP.

In conclusion, we have shown that the alkyl lysophospholipids examined here can exert transient stimulatory effects on PLD-mediated PtdCho hydrolysis as well as lasting inhibitory effects on PMA-induced phospholipid hydrolysis and DNA

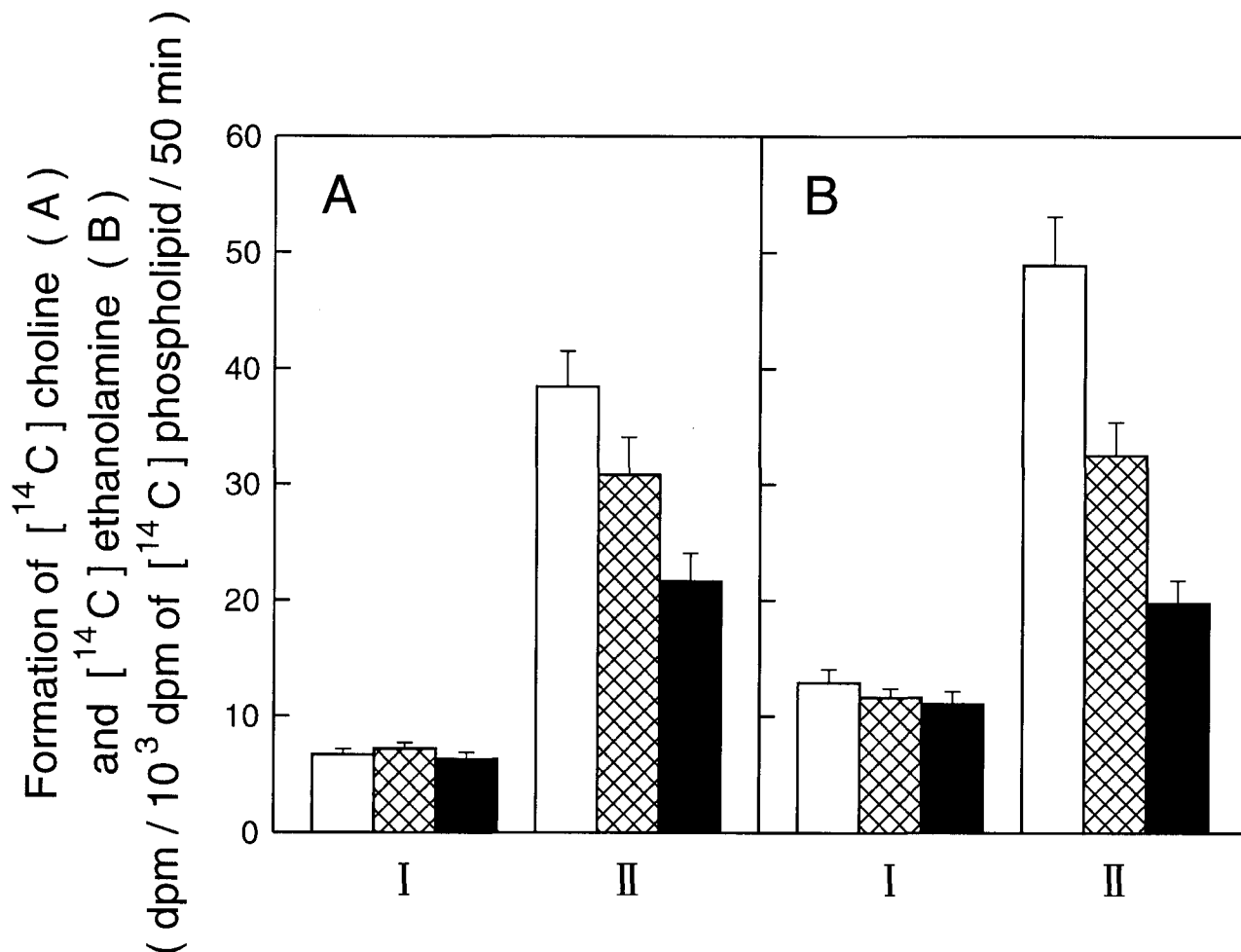


Fig. 3. Effects of BM41.440 on PtdCho and PtdEtn hydrolysis determined after a 3 h lag period following treatments with BM41.440. NIH3T3 fibroblasts were labeled with $[^{14}\text{C}]$ choline (A) or $[^{14}\text{C}]$ ethanolamine (B) for 48 h. Cells were washed and then incubated in serum-free medium for 30 min in the absence (open bars) or presence of 1 $\mu\text{g/ml}$ (cross-hatched bars) or 2.5 $\mu\text{g/ml}$ (filled bars) of BM41.440. Cells were washed and then incubated for 3 h in fresh serum-free medium. Finally, cells were washed again and then incubated (in serum-free medium containing either 20 mM choline or 2 mM ethanolamine as appropriate) for 50 min in the absence (I) or presence (II) of 100 nM PMA. Data represent the mean \pm SD ($n=4$) in a single experiment representative of three.

synthesis. Prolonged incubation of ALP-treated cells was found to increase the inhibitory potency of ALP. This would be consistent with the mediation of inhibitory ALP effects by a metabolite formed from ALP. Indeed, 1-*O*-hexadecyl-2-acetyl-sn-glycerol, which is known to be formed by PLC-mediated hydrolysis of ET-18-OCH₃, was shown to be a potent inhibitor of PKC [34–36]. We are presently investigating the possibility that NIH 3T3 cells are also able to form a similar PKC inhibitor from BM41.440. Alternatively, time-dependent redistribution of ALP to intracellular membranes containing the PMA-inducible PLD activities could explain the lag peri-

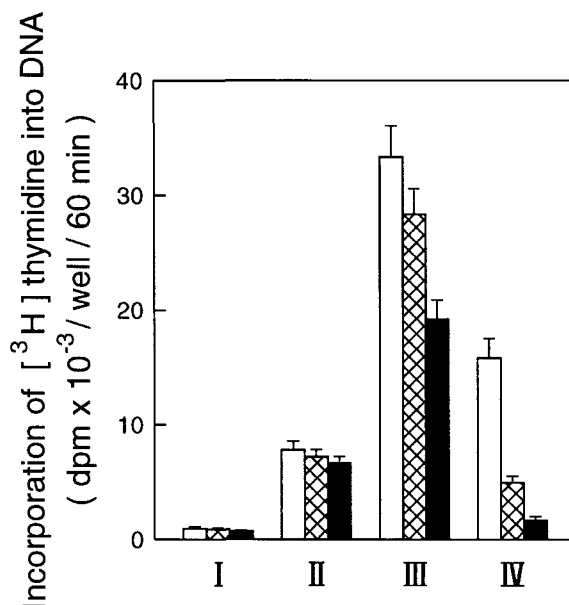


Fig. 4. Effects of BM41.440 on DNA synthesis induced by PMA and other mitogens. Serum-starved (24 h) NIH 3T3 fibroblasts were incubated (in serum-free medium) for 30 min in the absence (open bars) or presence of 1 $\mu\text{g/ml}$ (cross-hatched bars) or 2.5 $\mu\text{g/ml}$ (filled bars) of BM41.440. Cells were washed and then incubated (in serum-free medium) for 16 h in the absence of other additions (I) or in the presence of 500 nM insulin (II), 1 mM phosphocholine plus 1 μM sphingosine-1-phosphate (III), or 100 nM PMA. Data represent the mean \pm SD ($n=3$) in a single experiment representative of three.

od needed to enhance the inhibitory potency of ALP. PMA-induced mitogenesis was also preferentially inhibited by low concentrations of ALP. This is a further indication that the PKC system is likely to be one of the primary targets of ALP. It remains to be determined if and how the effects of ALP on the PLD and PKC systems relate to their antineoplastic effects.

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